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Research Paper

Enho Mutations Causing Low Adropin: A Possible Pathomechanism of MPO-ANCA Associated Lung Injury

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ABSTRACT

Background: Myeloperoxidase (MPO) anti-neutrophil cytoplasm autoantibody (ANCA)-associated vasculitis commonly causes life-threatening pulmonary alveolar hemorrhage or fibrosis. Only a limited number of candidate gene variants have been explored, but hitherto, are not widely confirmed. In the present study, we investigated the importance of energy homeostasis associated gene (*Enho*) mutations and adropin deficiency in the development of MPO-ANCA associated lung injury.
Methods: We analyzed the peripheral blood mononuclear cells from 152 unrelated patients and 220 population-matched healthy individuals for genetic variations in *Enho*. Functional studies with adropin knockout (AdrKO) on C57BL/6J mice were also performed.
Findings: Sequencing revealed six patients with p.Ser43Thr and that five patients shared Cys56Trp amino acid substitution in *Enho*. Serum concentration of adropin was significantly lower in patients than that of the healthy subjects ($P < 0.0001$), especially those with *Enho* mutations. *In vivo*, homo- and heterozygous carriers of the null adropin allele exhibited MPO-ANCA associated pulmonary alveolar hemorrhage as compared to wild-type mice. AdrKO mice exhibit reduced eNOS (Ser1177) and Akt1 (Ser473) phosphorylation and loss of T_{reg} cells.
Interpretation: Our findings indicate that the presence of *Enho* mutations or adropin-deficiency is a probable molecular basis for the initial events triggered in MPO-ANCA associated lung injury.

1. Introduction

Anti-neutrophil cytoplasm autoantibody (ANCA)-associated diseases are autoimmune conditions characterized by necrotizing inflammation of small blood vessels with significantly higher mortality rates than other autoimmune diseases (Jones et al., 2010; Nakaya et al., 2013). In ANCA-associated vasculitis (AAV), particularly in myeloperoxidase (MPO)-specific ANCA-positive cases, the clinical studies have been mainly focused on renal lesions (Jennette and Falk, 2014). However, it has become clear that pulmonary lesions such as alveolar hemorrhage or fibrosis appear concurrently to renal lesions (Zhang et al., 2014). Furthermore, there is *in vitro* as well as *in vivo* evidence to suggest a potential pathogenic role of ANCA in pulmonary vasculitis (Falk et al., 1990; Holguin et al., 2008), but the pathomechanisms are yet unidentified. Additionally, a debate has ensued about whether it is an autoimmune syndrome of a single disease entity or distinct between proteinase 3 (PR3)-AAV and MPO-AAV (Lyons et al., 2012; Hogan et al., 2006).

Several studies provide evidence of potential genetic contribution towards AAV (Knight et al., 2008, Monach and Merkel, 2010). The most convincing association has been with the major histocompatibility

Abbreviations: AAV, ANCA-associated vasculitis; ANA, anti-nuclear antibodies; ANCA, anti-neutrophil cytoplasmic antibody; ELISA, enzyme-linked immunosorbent assay; MPO, myeloperoxidase; PR3 or *PRTN*3, proteinase 3; MHC, major histocompatibility complex; *SERPINA*1, serpin *A*1 gene; *Enho*, energy homeostasis associated; AdrKO, adropin knockout mice; eNOS, endothelial nitric oxide synthase; RNA-seq, Transcriptome deep sequencing; CRISPR, clustered regularly interspaced short palindromic repeats; EC, endothelial cells; CRP, C-reactive protein; TNF-α, tumor necrosis factor alpha; AECA, anti-endothelial cell antibody; OPN, osteopontin; ET-1, endothelin-1; AdrHET, Heterozygous males and females mice; DAPI, 4′, 6-diamidine-2-phenylindole dihydrochloride; VCAM-1, vascular cell adhesion molecule-1; COPA, Coatomer subunit alpha; STING, stimulator of interferon genes.

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complex (MHC), especially the locus *HLA DPB1*0401* (Wieczorek et al., 2010, Jagiello et al., 2004). The other has been suggested between AAV and the rare Z (or null) allele of the serpin *A*1 gene (*SERPINA1*) that encodes α1-antitrypsin, a serine proteinase inhibitor for PR3 (Monach and Merkel, 2010; Morris et al., 2011). Both, the *HLA-DP* and *SERPINA1* associations are observed unambiguously in granulomatosis patients with polyangiitis, also positive for PR3-ANCA, but not for MPO-AAV. Thus, the genetic etiology leading to MPO-AAV or MPO-ANCA associated lung injury has remained elusive.

Adropin, a product of the energy homeostasis associated gene (*Enho*), is a recently identified protein that has been implicated in insulin resistance (Aydin et al., 2013; Kumar et al., 2008) and as a novel regulator of endothelial function (Goetze and Albrethsen, 2014; Lovren et al., 2010). It has been reported that adropin exhibits a potential role to protect endothelium by upregulating endothelial nitric oxide synthase (eNOS) expression through the VEGFR2-PI3K-Akt pathways (Lovren et al., 2010; Ganesh Kumar et al., 2012; Kessenbrock et al., 2009). Our preliminary study found, human MPO-ANCA-related pulmonary hemorrhage displaying adropin-deficiency and target genes mutations. Therefore, we hypothesized that adropin may also exert direct effects on the endothelium in AAV and MPO-ANCA associated lung injury.

2. Materials and Methods

2.1. Study Population

We conducted a chart review for patients who were diagnosed with MPO-ANCA-related vasculitis during hospitalization from February 2013 through November 2015 at the 1st Affiliated Hospital, 2nd Affiliated Hospital, Union Hospital, Mindong Affiliated Hospital, and Quanzhou 1st Affiliated Hospital, Fujian Medical University; Xiamen Hospital of T.C.M; Affiliated Hospital (Group) of Putian University; China (Supplementary Fig. 1). MPO-ANCA-related pulmonary vasculitis cases were further defined by the following inclusion criteria: positive MPO-ANCA serology, open or thoracoscopic lung biopsy, evidence of vasculitis in the histology, and negative serum glomerular basement membrane antibodies. All patients were evaluated for the presence of autoimmune antibodies including anti-nuclear antibody (ANA), MPO-ANCA, and PR3-ANCA. According to the Japanese guideline for idiopathic interstitial pneumonias, basically all interstitial pneumonia cases are recommended to examine serum ANA, MPO-ANCA, PR3-ANCA and other autoimmune antibody as a routine screening test to exclude the collagen vascular disease from idiopathic interstitial pneumonias. In the guideline, the serum MPO-ANCA level of >20 EU is suggested to be positive. There were 152 patients included, with female predominance (female: male ratio approximately 3:2), age from 39 to 72 years, and the median age was 52.5 years old. What's more, 30 cases of pulmonary bulla (aged from 25 to 60 years old, median age 37.6 years), 34 cases of pathologically confirmed lung cancer (age from 42 to 73, median age 57.5 years), 40 cases with pneumonia (age from 36 to 70, median age 55.3 years) were included, and 220 healthy controls (aged from 35 to 60 years old, with a median age of 47.6 years) were serving as controls. This research project was approved by the Ethics Committee of the Fujian Medical University, which supervised the study.

2.2. Analysis of Gene Mutations

Informed consent was obtained from patients and healthy donor controls. Blood was collected and DNA extracted using a Tiangen Genomic extraction kit (Beijing, China). Full-length *Enho* was amplified, purified, and sequenced.

2.3. Gene Targeting in AdrKO Mice

AdrKO mice were generated by clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 by the Shanghai Biomodel Organism Science & Technology Development Co., Ltd. on the C57BL/6J background. Heterozygous males and females (AdrHET) were then mated to produce homozygous carriers of the null *Enho* allele (AdrKO). All animal experimental procedures were approved by the Committee, use of Live Animals for Teaching and Research at Fujian Medical University and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals. AdrKO mice and wild-type littermates (WT) were housed in a 12 h light or dark cycle room under controlled temperatures (23 ± 1 °C) with free access to water and standard chow (20% kcal protein, 10% kcal fat, and 70% kcal carbohydrates).

2.4. Reference Multi-testing Algorithm

Serum levels of adropin, C-reactive protein (CRP), tumor necrosis factor alpha (TNF-α), anti-endothelial cell antibody (AECA), osteopontin (OPN), endothelin-1 (ET-1), and MPO from AdrKO, AdrHET, and WT mice were measured using a specific enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's protocols.

2.5. RNA-seq

Transcriptome deep sequencing (RNA-seq) was performed using total RNA isolated from lung tissue of three AdrKO and three age-matched littermates (male F2 intercross mice). Three individuals from each genotypic group were randomly selected. Total RNA was extracted from frozen tissue using the SV Total RNA Isolation System (Promega Corporation, Madison, WI) according to the manufacturer's instructions. The quantity and quality of RNA samples were assessed by Nanodrop 1000 (Thermo Fisher Scientific Inc., Wilmington, DE, USA). Total RNA samples were sent to DRIGEN Co., Ltd. for RNA-seq library preparation using the TruSeq SBS Kit (75 Cycles) and single end sequencing by means of an Illumina NextSeq 500 machine (Illumina). RNA-seq reads were quality filtered using SolexaQA packages with default parameters and a filter for the requisite length greater than 70 bp for both ends of each read pair. Sequencing data have been submitted to the NCBI Sequence Read Archive.

Quality filtered RNA-seq reads were mapped to the mouse reference genome, mm10, with TopHat v2.1.0. The comparisons between treated and normal mice were made using custom Perl scripts. Genes that showed significant ($P < 0.05$) difference in transcript levels were termed as differentially expressed (DE) genes.

2.6. Histology and Immunohistochemistry

Lungs tissue were fixed in 4% formalin overnight, embedded in paraffin, sectioned at 4 mm and stained with hematoxylin and eosin (H&E) for pathology. The following antibodies were used: OPN (ABclonal, A1361), CD3 (ABclonal, A1753), CD20 (ABclonal, A1793), CD38 (ABclonal, A1680), Ki-67 (ABclonal, A18522), CD31 (ABclonal, A31811), and VCAM-1 (ABclonal, A0279).

2.7. Western Blot Analyses

Proteins from lungs of AdrKO and age-matched littermates were separated on 4 to 12% Tris-glycine gels and transferred to nitrocellulose membranes. Membranes were probed with antibodies directed against (phospho-) eNOS (ABclonal, A1548), total eNOS (ABclonal, A1796), (phospho-T450) Akt1 (ABclonal, AP0004), (phospho-S473) Akt1 (ABclonal, AP0140), Mapk1 (ABclonal, A0229), and Gapdh (ABclonal, AP5809).